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# Distribution and stability of supernumerary microchromosomes in natural populations of the Amazon molly, *Poecilia formosa*

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**Abstract.** In animals, supernumerary chromosomes and their evolution have mostly been studied in sexual reproducing species. In the present study, for the first time, the natural distribution and stability of supernumerary microchromosomes were investigated in the unisexual fish species *Poecilia formosa*. Natural habitats throughout the range of *P. formosa* were screened for the presence of microchromosomes over several years. A high frequency of microchromosomes was found in the Río Purificación river system. Evidence points to the presence of the same microchromosome lineage over many generations. No supernumerary chromosomes were found elsewhere than in the Río Purificación representing a significant difference in the distribution of microchromosome-bearing individuals between the Río Purificación and all other collection sites.



Supernumerary chromosomes, also called B chromosomes, have been found in all major groups of plants and animals (Jones and Rees, 1982). Their occurrence in fishes has been described earlier. In addition to the 21 fish species listed by Salvador and Moreira-Filho (1992), five other species have been reported to have supernumerary chromosomes (Vicente et al., 1996). They vary greatly in size from microchromosomes (e.g. in *Prochilodus scrofa*, Pauls and Bertollo, 1983; and *Moenkhausia sanctaefilomenae*, Foresti et al., 1989), to medium-sized chromosomes (e.g. *Rhamdia hilarii*, Fenocchio and Bertollo, 1990) or even macrochromosomes (*Astyanax scabripinnis*, Maistro et al., 1992; *Alburnus alburnus*, Ziegler et al., 2003).

Two primary sources for B chromosomes have been considered (Jones and Rees, 1982; Green, 1990; Camacho et al., 2000): either an intragenomic fragment acquires the characteristics of a B chromosome from duplicated or fragmented pieces within a genome, or interspecific hybridization provides foreign DNA from a closely related species that evolves into a supernumerary chromosome.

*P. formosa*, commonly called Amazon molly, is a small freshwater fish distributed in southern Texas and northeastern Mexico (Schlupp et al., 2002). It was the first unisexual vertebrate to be described (Hubbs and Hubbs, 1932). Its mode of reproduction is gynogenesis (Kallman, 1962) which is defined as sperm-dependent parthenogenesis. Normally, females produce unreduced diploid eggs which are only activated for embryogenesis by sperm of males of closely related species (*P. mexicana*, *P. latipinna*, *P. latipunctata*, Schlupp et al., 2002). Therefore, the offspring is genetically identical to the mother. Supernumerary chromosomes in this species most probably result from a failure in the mechanism, which clears the egg from the sperm nucleus. The interspecific origin could be demonstrated for individuals who expressed a paternal

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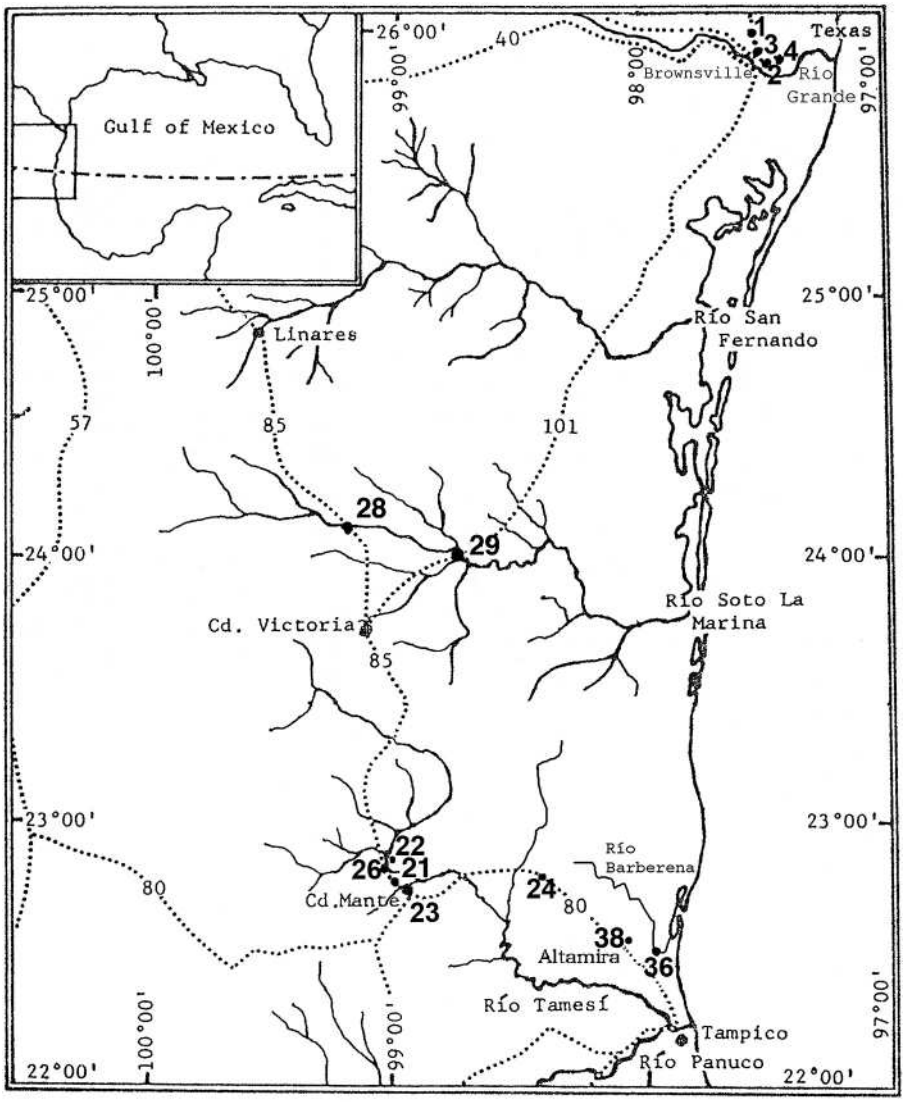


**Fig. 1.** Map of the different collection sites in northeastern Mexico and southern Texas as listed in Table 1. Spotted lines are streets (see Balsano et al., 1972). San Marcos (not shown in map) is an introduced population from Caldwell County in central Texas. Source of the introduction was Brownsville (Schlupp et al., 2002).

macromelanophore locus resulting in spotted individuals (Schartl et al., 1995, 1997). Macromelanophores are a pigment cell type specific to the Black molly, an ornamented *Poecilia* strain selected for its black body pigmentation. Such Black mollies are commonly used as host males in the laboratory. This is a very rare situation since normally supernumerary chromosomes are considered to be genetically inert (Jones and Rees, 1982). In the Amazon molly, not only the above described microchromosomes of Black molly origin exist, but also naturally occurring microchromosomes in wild-type individuals have been seen (Sola et al., 1993).

In the genus *Poecilia* the karyotype usually shows 46 subtelocentric and acrocentric chromosomes (for an overview see Prehn and Rasch, 1969; Haaf and Schmid, 1984; Sola et al., 1992a; Schartl et al., 1995; Rodionova et al., 1996). In *P. formosa* different chromosomal clones have been described, mostly based on different nucleolus organizer region (NOR) positions (Sola et al., 1997), or heteromorphism of the short arms of chromosome pair 1 (Sola et al., 1992b).

In the present study the natural distribution and the stability of microchromosome bearing *P. formosa* lineages was studied over several years. We screened natural habitats throughout the range of the Amazon molly for the presence of microchromosomes. Animals bearing microchromosomes were bred for one generation to study whether the microchromosomes are transmitted stably to their offspring. We found a high frequency of microchromosomes in the Río Purificación river system, whereas in other parts of the natural range of *P. formosa* this phenomenon appears to be rare or absent. Evidence was obtained that points towards the presence of the same microchromosome over many generations.

## Materials and methods

*Fishes*

All experimental fish were kept in the aquarium of the Biocenter of the University of Würzburg under standard conditions. Wild fish were from the different collection sites in northeastern Mexico and Texas (Table 1, Fig. 1).

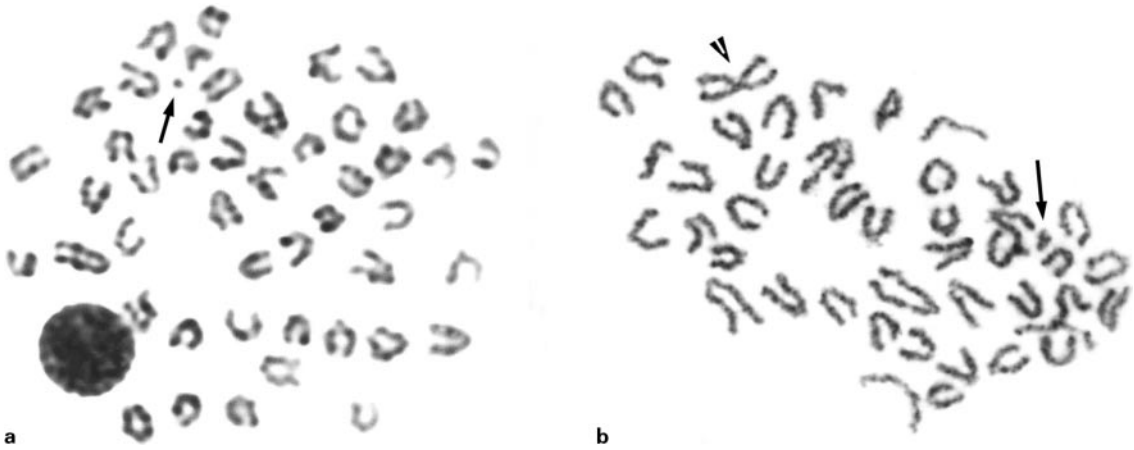


**Fig. 2.** Representative Giemsa-stained metaphases showing the presence of a single accessory (**a**) tiny and (**b**) large microchromosome in specimens of unisexual Amazon molly from the Río Purificación population. Arrows indicate the microchromosomes. Note the presence of a large metacentric chromosome (arrowhead) in 2n = 45 specimens with the larger microchromosome in **b**.

*Cytogenetic analyses*

Cytogenetic analyses were carried out according to Nanda et al. (1995). Chromosomes were Giemsa-stained. At least 10 Giemsa-stained metaphases were counted for each individual.

*Multilocus DNA fingerprinting*

DNA was extracted using EDTA buffer and phenol/chloroform according to the method of Blin and Stafford (1976). *Hin*fI was used for restriction digestion, and the restriction fragments were separated on a 0.8% agarose gel at 1 V/cm. In-gel hybridization was done essentially as described by Nanda et al. (1988). The $^{32}$P-labeled oligonucleotides $(GATA)_4$, $(GGAT)_4$, $(GA)_8$, and $(CA)_8$ specific to hypervariable simple repeats were used as hybridization probes.

## Results

A total of 129 individuals collected between 1993 and 2002 from 14 different sites were analyzed (Fig. 1). In addition to karyotypically normal (2n = 46) diploid specimens (N = 101), metaphases from 28 individuals showed a microchromosome. All metaphases analyzed from these microchromosome-carrying specimens conspicuously had a single microchromosome (Fig. 2). Thus the microchromosome appears to be mitotically stable. Further detailed analysis uncovered that some of these microchromosome-bearing individuals had one unpaired large metacentric chromosome and their diploid number was 45 instead. Due to its small size, it was not possible to detect specific features of the microchromosome using traditional cytogenetic methods. Intriguingly, microchromosomes were only found in females from the Río Purificación. Out of 28 females carrying microchromosomes, 7 individuals showed the normal 2n = 46 chromosome complement with one tiny microchromosome (2n = 46+m) (Fig. 2a). Fourteen individuals, however, were pseudoaneuploid with a diploid chromosome number of 45, and their karyotype displayed one large metacentric chromosome and a larger microchromosome (2n = 45+F+M) (Fig. 2b). Seven individuals showed a triploid karyotype with one tiny microchromosome of the same type as in the diploid karyotype (3n = 69+m). The frequency of microchromosomes in the Río Purificación (28 with and 47 without, 37.33%) differs significantly from the frequency at all other collection sites (0 with and 54 without) ($\chi^2$ test, df = 1, $\chi^2 = 23.61$, $P < 0.0001$; cf. Table 1). Individuals carrying microchromosomes or triploids do not differ phenotypically from diploid individuals.

Multilocus DNA fingerprinting of the microchromosome-bearing fishes revealed that one analyzed female showing the tiny microchromosome (2n = 46+m) belongs to clone *f* (Fig. 3), a frequent clone among diploid *P. formosa* (Lampert et al., 2004). This means that either a fish from clone *f* has acquired the microchromosome, or alternatively most of the fish of clone *f* have lost it.

Surprisingly, eleven analyzed females possessing the larger microchromosome (45+F+M) belong to six different clones. Clone *b′* shows one additional band to the overall identical banding pattern of clone *b,* whereas *c′* differed from clone c in lacking only one band (Fig. 3). Overall, the basic banding pattern of all clones is quite similar. These clones could not be found in 39 additionally analyzed diploid individuals (data not shown). Two triploid individuals with the tiny microchromosome were analyzed by multilocus DNA fingerprinting. They resemble two different clones (*C* and *E*). Whereas clone *D* is represented by this individual only, clone *C* is frequent among triploid animals (Lampert et al., 2004).

One female of each microchromosome-bearing clone was mated to a Black molly male, and the offspring was analyzed cytogenetically for the presence of the different microchromosomes. It could be shown that the females of the three different clones (2n = 46+m, 2n = 45+F+M, 3n = 69+m) transmitted their microchromosomes to the next generation (N = 5, N = 1, and N = 4, respectively).

**Table 1.** Number and type of microchromosomes found in *P. formosa* from natural populations in northeastern Mexico and southern Texas. 2n = 46 refers to animals without microchromosomes. 2n = 46+m and 3n = 69+m refer to diploid and triploid specimens with a single tiny microchromosome, respectively. Animals showing the large metacentric chromosome and a larger microchromosome are referred to as 2n = 45+F+M. Note that microchromosomes were only found in the Rio Purificación populations. Numbers of collection sites as given in Fig. 1.

| | Locality | Collection site no. | Year | 2n = 46 | 2n = 46+m | 2n = 45+F+M | 3n = 69+m | N |
|---|---|---|---|---|---|---|---|---|
| Mexico | Rio Purificación, Barretal | 28 | 1993 | 2 | 2 | 1 | – | 5 |
| | | | 1996 | 29[a] | 3 | 12 | – | 44 |
| | | | 1998 | 8 | – | 1 | 5 | 14 |
| | | | 2002 | 3 | 2 | – | 1 | 6 |
| | Rio Purificación, Nuevo Padilla | 29 | 1993 | 1 | – | – | – | 1 |
| | | | 1996 | 3 | – | – | – | 3 |
| | | | 1998 | 1 | – | – | 1 | 2 |
| | Rio Barberena, near Lomas del Real | 36 | 1994 | 2 | – | – | – | 2 |
| | Rio Guayalejo, near El Limón | 22 | 1993 | 1 | – | – | – | 1 |
| | Mante | 23 | 1993 | 1 | – | – | – | 1 |
| | Ditch north of Mante | 21 | 1993 | 2 | – | – | – | 2 |
| | | | 1998 | 5 | – | – | – | 5 |
| | near González (Mex 80) | 24 | 1993 | 3 | – | – | – | 3 |
| | Rio Guayalejo (Mex 85) | 26 | 1998 | 1 | – | – | – | 1 |
| | Laguna Champaxan near Altamira | 38 | 1994 | 3 | – | – | – | 3 |
| | | | 2002 | 8 | – | – | – | 8 |
| Texas | Northmost | 1 | 1994 | 1 | – | – | – | 1 |
| | Brownsville | 2 | 1994 | 5 | – | – | – | 5 |
| | Olmito | 3 | 1995 | 2[a] | – | – | – | 2 |
| | Bay View | 4 | 1995 | 2 | – | – | – | 2 |
| | San Marcos | | 1994 | 18[a] | – | – | – | 18 |

[a] Heteromorphism chromosome 1.

## Discussion

In the genus *Poecilia* the karyotype usually shows 46 subtelocentric or acrocentric chromosomes (Prehn and Rasch, 1969; Haaf and Schmid, 1984; Sola et al., 1992a; Rodionova et al., 1996). In cytogenetic studies on *P. formosa* only very small sample sizes have been analyzed up to date. Different chromosomal clones have been described in *P. formosa*, mostly based on different NOR positions (Sola et al., 1997), or heteromorphism of the short arms of chromosome pair 1 (Sola et al., 1992b). Sola et al. (1993) described a single individual out of six investigated fish from the Río Purificación, Nuevo Padilla, Mexico, showing an unpaired metacentric chromosome and a larger microchromosome. In addition, the authors found a triploid individual also showing a single microchromosome.

We report here, for the first time, on an extensive screening of different populations of *P. formosa* focusing on microchromosomes. It is noteworthy that we found the single tiny microchromosome in four different sampling years. No supernumerary chromosomes were found elsewhere than in the Río Purificación. Obviously, there is a significant difference in the distribution of microchromosome-bearing individuals between the Río Purificación and all other collection sites. Thus the Río Purificación may represent a hot-spot for the presence of microchromosomes. Several reasons may explain this pattern: the probability of de novo origin of microchromosomes at other sites may be very low, and/or the conditions for the persistence of such clones may be exceptionally favorable in the Río Purificación. Alternatively, it cannot be excluded that future samplings may reveal the existence of microchromosome-bearing clones at other sites.

As analyzed by multilocus DNA fingerprinting, the eleven females possessing the larger microchromosome (45+F+M) belong to six different clones. Taking into account that a similar banding pattern could not be found among 39 diploid individuals analyzed additionally, it is likely that this karyotype evolved only once, and that the observed intraclonal genetic differences are due to mutations that occurred afterwards. The existence of six clearly distinguishable clones points to the fact that many generations must have passed since their common origin because, in general, the mutation rate apparent in different DNA fingerprint patterns is very low in *P. formosa*.

Finding these different clones in subsequent years in the Río Purificación suggests them to be a stable component of the population. In addition, we could show that all offspring tested from the following generation invariantly had the same karyotype as their mothers, including the tiny microchromosome or the metacentric chromosome plus the larger microchromosome.

In summary, our study provides some information of the distribution and stability of accessory chromosomes in the genome of the asexual Amazon molly in natural condition. It is

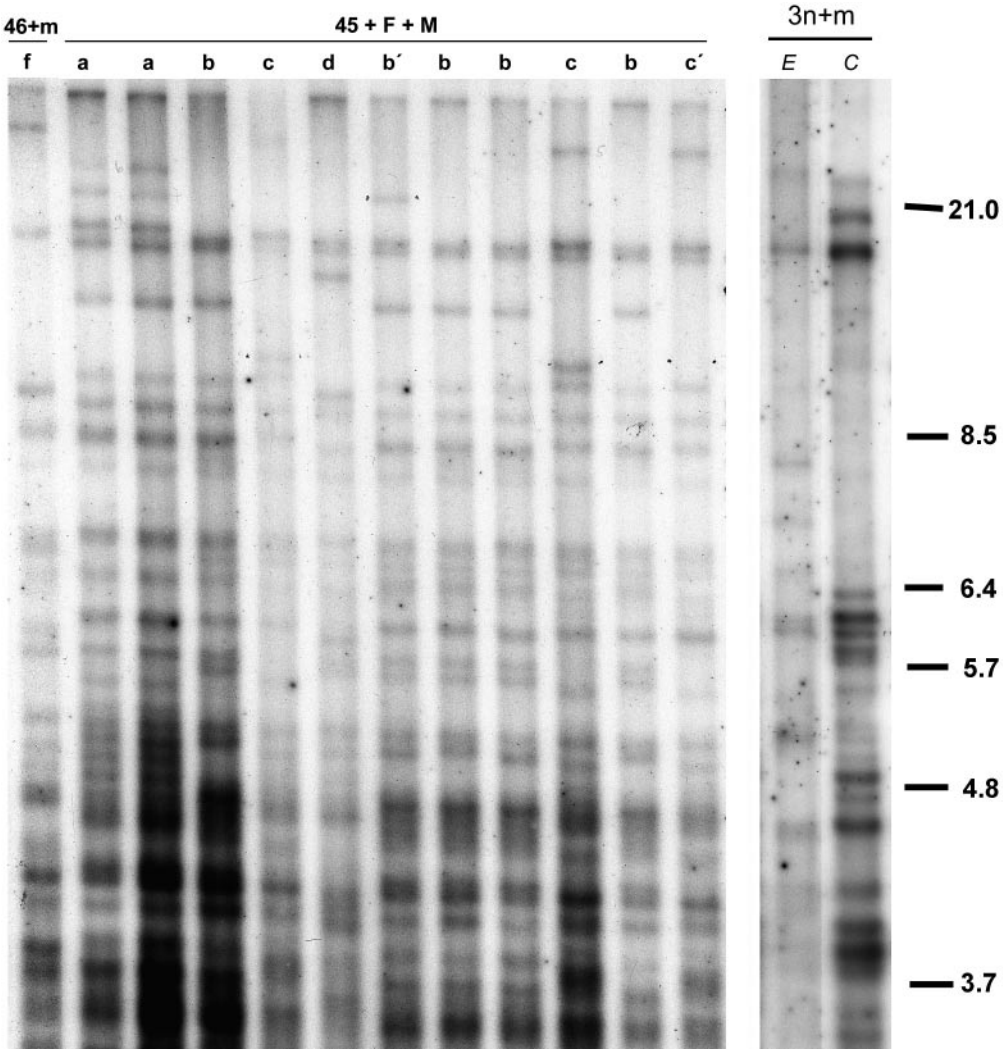


**Fig. 3.** Multilocus DNA fingerprint of one diploid animal with one tiny microchromosome (2n = 46+m) belonging to the common diploid clone *f* also found in animals without microchromosomes. Eleven individuals with the unpaired metacentric fusion chromosome and the larger microchromosome (45+F+M) making up six different clones with a very similar basic banding pattern. In-gel hybridization with $^{32}$P-labeled oligonucleotide $(GGAT)_4$. Two triploid individuals with one tiny microchromosome (3n = 69+m). Whereas clone *E* is made up of this single individual only, clone *C* is frequent among triploids. In-gel hybridization with $^{32}$P-labeled oligonucleotide $(GT)_8$.

noteworthy that our repeated sampling over extended years (1993–2002) failed to detect individuals with two or more microchromosomes. This is quite different from the situation reported for those from laboratory broods using the Black molly as sperm donor (Schartl et al., 1995). This might suggest the existence of an upper limit for B number under natural conditions.

The larger microchromosome in the fish with 2n = 45 karyotype can be explained by two possibilities. Either it is a host species-derived element like the other – although smaller – microchromosomes, or it could be of intragenomic origin. The appearance of a large metacentric chromosome along with a larger marker chromosome among the individuals with 2n = 45 can be assumed to be the result of a centric fusion involving a breakpoint within or near the centromeric region of two different chromosomes with a recognizable short arm. In this scenario the newly rearranged karyotype will contain a large monocentric metacentric chromosome (F) and consequently will gain a small centric translocation product (White, 1973; Holmquist and Dancis, 1979). In most instances such a small translocation product becomes unstable and is eventually lost due to the lack of a homologous partner during meiotic division in sexually reproducing animals. Absence of normal meiosis among asexually reproducing vertebrates like the Amazon molly may render to retain this small marker chromosome. In this regard, a fluorescence in situ hybridization experiment with a microdissected microchromosome paint is necessary for precise detection of the breakpoints preceding the centromeric fusion, and to prove that the small microchromosome indeed is an intragenomic segment.

In asexual (clonal) organisms mutations are usually the only source of genetic variability. In the case of the Amazon molly, microchromosomes play a role as an additional source of genetic variability, but their function and evolutionary significance remain to be tested.


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